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The Effect of Alloxan on the Pancreas, Liver and Kidney of the Teleost, *Lebistes reticulatus*, with Notes on the Normal Pancreas.

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(Plates I-IV).

CONTENTS.

INTRODUCTION.

In 1937 Jacobs reported that rabbits given an intravenous injection of alloxan suffered a hyperglycemia which is followed by a marked and often convulsive hypoglycemia. He alleviated the hypoglycemic state by glucose administration but made no histological studies of the pancreas. Dunn, Sheehan & McLetchie (1943), while studying the effects of a number of nephrotoxic drugs in rabbits, included the compound. They confirmed the pronounced blood sugar level changes and in addition reported extensive damage to the insulinogenic beta cells of the islands of Langerhans. Dunn & McLetchie (1943), using rats, continued the work and showed that the hypoglycemic state is temporary and is very soon followed by a permanent hyperglycemia. They considered the blood sugar level changes to be a physiological reflection of the demonstrated cytological alterations of the beta cells. That the effects are not due to an insulin inactivation by alloxan or to the alloxan per se, has been shown by Goldner & Gomori (1944a, 1944b), Kennedy & Lukens (1944), and Corkill *et al* (1944).

Following these reports, alloxan studies have been extended to include many different animals, among which are the following: dog (Goldner & Gomori, 1943; Houssay, Brignone & Mazzocco, 1946), cat (Ruben & Yardumian, 1946), sheep (Jarrett, 1946), monkey (Banerjee, 1944), pigeon (Goldner & Gomori, 1945), duck (Mirsky, 1945), turtle (Ramos, 1944), frog (Seiden, 1945), toad (Biasotti & Porto, 1945), elasmobranch and marine teleosts (Saviano, 1947a, 1947b). It has been found that all of these animals are affected in some degree by alloxan, and may show either histological or physiological changes, or both.

Following the administration of a diabetogenic dose of alloxan there occurs a characteristic tri-phasic blood sugar level curve. An initial rise in blood sugar at 2-4 hours after the drug administration, a secondary marked and often fatal hypoglycemia after 6-12 hours, finally a permanent hyperglycemia is noted (Duff & Starr, 1944; Houssay, Orias & Sara, 1945; Lazarow, 1946). That the curve may be tetra-phasic is indicated by the work of Shipley & Beyer (1947) who noted a slight initial drop in blood sugar level at 15-30 minutes after the alloxan administration.

In some mammals cytological changes in the beta cells have been reported to take place very rapidly. Hughes, Ware & Young (1944) claim that degranulation is detectable 5 minutes after a subcutaneous diabetogenic injection in the rat. In most animals studied, degranulation, nuclear pycnosis and cytolysis with an accompanying distortion of island architecture occurs within 24 hours. These changes, once established, are permanent.

It is claimed by some investigators that a number of complications and effects upon other organs accompany the diabetic state produced by the alloxan administration. Goldner & Gomori (1943), Kendall *et al* (1945) and Herbut *et al* (1946) report a fatty infiltration of the liver. Necrosis of this organ is recorded by Herbut *et al* (1946), Lazarow & Palay (1946), and Ruben & Yardumian (1946). Acute renal damage demonstrated histologically by vacuolation and desquammation of the convoluted tubules is reported by Dunn, Sheehan & McLetchie (1943), Bennett & Behrens (1946) and Jarrett (1946). Pancreatic acinar tissue injury is claimed by Di Pietro & Cardeza (1946) and Duff *et al* (1947). An extensive hemolysis is indicated by the work of Kennedy & Lukens (1944) and Gyorgy & Rose (1948). Although most investigators have found that the island damage is limited to the beta cells, there are reports that the island alpha cell type is also affected (Duff & Starr, 1944; Saviano, 1947).

In view of these findings, it became of interest to study the effects of alloxan upon

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the viscera of the teleost, *Lebistes reticulatus* Peters.

As a necessary preliminary to the alloxan study, the location and the cytology of the islet of Langerhans was determined. It has been known for many years that fishes possess the equivalent of the mammalian islands of Langerhans. It is usual for the islet tissue of a teleost to be condensed into a few, and sometimes into only one, nodule of a large size. The islets may in some species be so large that they are visible with the naked eye. Stannius in 1846 reported the presence of a variable number of bodies in the abdominal cavity of the bony fishes. He associated these with the lymphatic and circulatory systems, but from the description and location given it is now known that the structures are the islets of Langerhans. After Langerhans directed attention to cell aggregations in the pancreas of the rabbit in 1869, there were many attempts to find their homologue in other forms. Harris & Gow (1894), in a comparative study of the pancreas, neglected the fish pancreas because they doubted the existence of such a functional organ in the class. Laguesse in 1895 reported the presence of islet epithelial cells in the teleost *Crenilabrus melops* (Linnaeus). In the same year Diamare described in *Lophius piscatorius* Linnaeus a macroscopic nodule about the size of a pea. On histological grounds he considered it to be analogous (in a report 3 years later he used the term homologous) to the mammalian islands of Langerhans. In the same report he listed several smaller bodies having the same structure as the large body. The studies continued and in a few years it was shown that islet epithelial cells could be demonstrated, either macroscopically or microscopically, in a variety of species of both fresh water and marine teleosts.

That the islets of the teleosts are composed of different cell types was noted as early as 1898 by Massari. He reported that the nuclei of all the islet cells possess the same staining qualities, but that the cytoplasm of some of the cells is more chromophilic than in other islet cells. Jackson (1922), using the differential solubility method of Lane, concluded that the teleost islet consists essentially of alpha and beta cells. Bowie in 1924 demonstrated that the islet tissue of *Neomaenis griseus* (Linnaeus) consists of three types of granular cells; he termed these alpha, beta and gamma cells. In Bowie's report no mention was made of the presence or absence of the agranular "C" cell such as has been reported to be present in the guinea pig pancreas. In the present study it was thought that the use of the azan stain, which has been shown to be capable of demonstrating all islet cell types reported to exist, would yield some information on this important fundamental point. It was also expected that the alloxan study might supplement the cytological study of the normal islet.

The experimental work of this study was carried on in the laboratory of the Department of Fishes and Aquatic Biology of The American Museum of Natural History. The author expresses his appreciation to Dr. Charles M. Breder, Jr., and Miss Priscilla Rasquin for their valuable suggestions and criticisms of the manuscript. Thanks are due to Dr. Ross F. Nigrelli for his reading of the manuscript.

Materials and Methods.

Mature, well-fed, healthy, active *Lebistes reticulatus* were selected for the experiment. Three series of experiments were established in an attempt to determine the effect of different dosages, and if possible ascertain the upper sub-lethal dose and the minimal dose capable of producing islet cell changes.

In the first series of experiments 14 females were kept immersed in a 0.05% alloxan solution. This concentration was lethal to some and deaths occurred as fo'lows: 1 in the 0-10 hour interval, 2 in the 10-20 hour interval, 6 in the 20-30 hour interval. Two fish were sacrificed at the end of the first 10 and 20 hours. One, when found in a moribund condition at 40 hours, was sacrificed. Fourteen males were kept immersed in a 0.05% alloxan solution. Deaths were recorded as follows: 2 in the 0-10 hour interval, 2 in the 10-20 hour interval, 6 in the 20-30 hour interval. Two fish were killed at the end of the first 10 and 20 hours. All specimens were fixed in Bouin's fluid.

In the second series of experiments 14 females were kept immersed in a 0.025% alloxan solution. One death occurred; this was in the 30-44 hour interval. Two fish were sacrificed at 10, 20, 44 and 70 hours after the start of the experiment. Two were killed on the fifth and seventh days. All of these were fixed in Bouin's fluid. The one remaining fish was killed and fixed on the ninth day in Flemming's fluid. Fourteen males were kept immersed in a 0.025% alloxan solution. Two fish were killed at the 10, 20, 44 and 70 hour intervals. One fish was found dead at the 20 hour interval. Two fish were sacrificed on the fifth and seventh days. Bouin's fluid was used as a fixative for all of these. The last fish, for which Flemming's fluid served as the fixative, was sacrificed on the ninth day.

In the third series of experiments 14 females were kept immersed in a 0.013% alloxan solution. Two fish were killed after 10, 20, 44 and 70 hours. Two fish were sacrificed on the fifth and seventh days. Bouin's fluid served as the fixative for these. On the ninth day one fish was fixed with Flemming's fluid. The last fish was maintained until it died on the thirty-ninth day. Fourteen males received similar treatment. The last male died on the forty-first day.

The fish were kept in groups of seven in two-gallon aquaria, allowing 5 liters of solution for this number. Males were kept separate from the females. Tanks were maintained without plants or snails. Fresh alloxan solutions using conditioned water were made up semiweekly. For controls, mature, active

male and female fish of approximately the same size as the experimental animals were used. Both experimental and control fish were maintained on a diet consisting of approximately 12% protein, 2% fat and 32% carbohydrate. In addition, algae was available for the control fish; this growth was lacking in the experimental tanks. The temperature averaged 72°F., the high being 73°F., the low 69°F.

In preliminary tests for differentiating the various islet cells, Helly's, Zenker's, and Bouin's fluids were used as fixatives. It was found that Bouin-fixed tissue, washed in water, permitted a more highly specific coloration of the islet cells than tissue fixed in the other fluids tested. In the main part of this experiment, as has already been noted, Bouin's fluid was used exclusively, except in the four cases specified in which Flemming's fluid was used. The control fish were fixed in either Bouin's or Flemming's fluid. Fish to be fixed in Bouin's fluid were killed by placing them in the fixative; those to be Flemming-fixed were killed by cutting the spinal cord. The viscera were immediately removed and placed in the respective fluid. Tissue was left in Bouin's fluid for 8-10 hours, in Flemming's fluid for 24 hours, washed in water, passed through graded alcohols, xylol, and embedded in paraffin. Sections were cut at 4 and 5 microns. One of the two fish sacrificed at each time interval in each of the series, was stained by the Gomori modification of the azan stain; the other was stained with Harris' hematoxylin counterstained with eosin. Thus for every time interval (excepting the ninth day) in each series, one female and one male were stained with hematoxylin-eosin, one female and one male were stained with the azan stain. Harris' hematoxylin and eosin were used for the Flemming-fixed tissue. A number of the sections from fish killed at each time interval of each series were checked for the presence of hemosiderin with 2% potassium ferrocyanide and eosin.

To determine the position of the islet of Langerhans, serial sections were prepared. Fish were fixed using Bouin's fluid. To aid in penetration the abdominal wall was punctured so as to allow the fixative to flood the abdominal cavity quickly. Decalcification was accomplished by the phloroglucin-nitric acid method. The bodies were passed through graded alcohols, xylol, and embedded in paraffin. Cross, frontal and sagittal sections were cut at 5 microns. Staining was with Harris' hematoxylin and eosin.

Normal Morphology of the Pancreas.

The exocrine pancreas of *Lebistes reticulatus* is a diffuse type with the cells arranged in bands. The greater part of these are found in the mesenteric area about the anterior part of the intestine and the spleen; often they extend posteriorly to the limit of the abdominal cavity. In addition, intrahepatic acinar cells are found in some specimens. The endocrine epithelial pancreatic cells are separate from the exocrine pancreas and form one compact nodule. As a rule this islet lies slightly anterior to the spleen; occasionally it is found extending to that organ, so that the two are seen in the same cross section. It is almost always situated in the mesentery on the left ventral surface of the stomach above the liver as seen in Figs. 1 and 3 (sometimes it is on the median line as in Fig. 2). It is in the area in which are located the mesenteric and hepatic arteries, the portal and splenic veins. The median margin of the structure is often seen to be in contact with the bile duct or the gall bladder. In cross section the islet is either oval or elliptical in shape; the former shape is more commonly found. In sagittal and frontal sections it appears elliptical. Owing to its pale pink color and thickness, it may be see macroscopically in large specimens as a pin point against the more translucent surrounding tissue.

The islet is bounded by a delicate fibrous connective tissue capsule. Occasionally a partial encirclement by the exocrine pancreatic cells is noted; this is seen in Fig. 7. Very delicate supporting connective tissue trabeculae are visible in some sections. No large blood vessel is seen to penetrate the islet; a very abundant blood supply is received through arterioles from the adjacent large blood vessels. Large conspicuous capillaries are abundant but are not equally distributed throughout the organ. Near the median border they are so numerous that in many cases they are separated from one another by two, and in some sections one, cell thickness only. In this region the capillaries are nearly parallel. Because of this arrangement the cells between them appear to be in columns or bands.

In routine hematoxylin-eosin preparations the pancreatic acinar cells stain very deeply. The zymogen granules are found in the inner zone of the cell, while the spherical nucleus with a centrally placed nucleolus is situated at the base of the cell. The islet cells are smaller and stain lighter than the exocrine pancreatic cells. That more than one type of cell is present in the islet is suggested by nuclear differences. Although there is a slight difference in chromaticity of the cytoplasm in different areas, cytoplasmic structural differences which could be used as criteria for cellular differentiation are not visible in the hematoxylin-eosin stained sections. The cells which stain darker are largely restricted to the marginal border and are usually arranged in bands between the capillaries that are so numerous in the area. Because of this arrangement these cells have a richer blood supply than other islet cells. Cell boundaries are not very distinct, but from the nuclear arrangement the cells appear to be cylindrical or spindle-shaped. The nuclei of these cells are oval, stain darkly and are filled with numerous minute chromatin granules. The nucleolus is distinct and centrally placed. The slightly lighter-stain-

ing cells are irregularly polyhedral in shape and are larger than the darker-staining elements. The nuclei are situated nearer to one end of the cell, are almost always round and are larger than the previously discussed nuclei. One or two eccentrically placed nucleoli are found. The darker-staining elements are the beta cells, the lighter elements are the alpha cells. A third type of cell is indicated and is present almost exclusively at the periphery of the organ. The cytoplasm takes very little stain, is very clear, and often is visible only with optimum staining conditions. The nucleus of this cell type is very large and except for a centrally placed nucleolus is also clear. This is the "D" type of cell.

The presence of these three types of cells was verified and further differences between them made evident when the azan stain was employed. With this stain the beta cell boundaries became more distinct and the fusiform shape indicated by the hematoxylin-eosin stain was confirmed. The cytoplasm when relatively degranulated is bluish and often a very small agranular area is seen at one side of the nucleus. The granules of this cell type are minute and stain reddish. As the number of granules increase and become crowded the cytoplasm itself seems red. The oval nucleus containing a distinct nucleolus stands out very clearly in the center of the cell. Because of this nuclear disposition the granules have a bipolar arrangement. The cells are smaller than the alpha variety and do not show a great change in degree of granulation. The beta cells make up almost exclusively the median border of the islet but are not restricted to this area; a few are found scattered in other regions. At this border they are arranged in definite cords which branch and anastomose. As a rule the elements are at right angles to the long axis of the columns; this often permits two ends of a cell to reach a capillary. This is the cell type that underwent involution after alloxan administration. Normal beta cells are seen in Fig. 4. The cytoplasm of the alpha cells stains a rich orange-yellow color. Their coarse granules stain a reddish-orange; in size they are intermediate between the large zymogen granules of the pancreatic acini and the fine granules of the beta cells. The degree of granulation of these cells normally varies. The nucleus is eccentrically placed and contains little chromatin material. The "D" variety of cell, which is found along the islet periphery, has a cytoplasm which stains blue. The cellular outline is not always well defined, but it can be determined that this cell is larger than both the alpha and the beta cells. Granulation is very fine and a light blue. Cells containing a very small number of granules are numerous. The nucleus is large and densely chromatic. It is centrally placed and also stains blue; one distinct nucleolus is found. Agranular "C" type cells were not present.

A differential cell count was not made but a close examination gives the impression that the alpha cells are more abundant than the beta variety. The "D" cells make up the smallest percentage of islet cells.

Experimental Results.

In the three series of experiments the reactions of the females and males to alloxan were essentially the same. Most differences that did occur were no greater than those found between individual animals of the same sex who received identical treatment. To avoid repetition, males and females of the same series are grouped together; where differences existed they are listed separately. No difference in the histological detail of the exocrine or endocrine pancreas was noted between gravid and non-gravid females. Except where specifically noted, the alpha, "D" islet cells and the pancreatic acinar cells were uninjured.

Series I (0.05%):

Within an hour of placing the fish in the alloxan solution all the females went to and remained at the surface. The males acted similarly after a lapse of five hours. That they were able to swim and reach the bottom was proved by their doing so when the side of the tank was tapped. When quiet prevailed they again rose to the surface. It appeared that they suffered a respiratory distress and sought a greater oxygen supply.

The two males and the female found dead at the 10 hour period were not suitable for a histological study. The islets of three of the four fish sacrificed at this time showed a very definite increase in capillary spaces and beta cell nuclei chromaticity. With eosin the cytoplasm of these cells stained more homogeneously than that of normal cells. Granulation was little changed and could be seen in all cells. The islet of the fourth fish, a female, showed an increase in vascularity and had a few deep-staining nuclei. No change was noted in any other organ.

In the 10-20 hour interval two females and two males were found dead. Microscopic examination showed advanced decomposition, indicating they had been dead for a number of hours. They were not suitable material for study. The islet of each of the four fish sacrificed at this time presented a much more advanced stage of degeneration than at the 10 hour interval. High power examination revealed a very intense hyperchromasia and condensation of the beta cell nuclei; in some areas nuclear fragmentation was also apparent. Degranulation in various degrees was evident; a range from complete degranulation to an almost normal complement was seen. Cellular arrangement was modified and continuity of the columns of cells was interrupted by shrinkage of cells. Liver parenchyma was normal. Hemosiderin in amounts greatly exceeding the normal was found in the liver and in the kidney. The latter organ was in all other respects histologically normal.

By 30 hours six females and six males had died; these were discarded because of decom-

position. At 40 hours a moribund female fish was sacrificed. Most of the beta cells were completely disintegrated, in some sections only an eosinophilic cellular debris remaining. A few remaining cells became rounded and shrunken. These were grouped in small numbers. In these cells the cytoplasm was almost colorless and completely degranulated and their nuclei were very deep-staining. The pancreatic acinar cells showed a lighter staining cytoplasm than normally seen. A few of these cells were vacuolated. The liver at this time presented small localized lesions. In some areas of this organ only shrunken parenchyma cells and a connective tissue stroma were seen. Occasional vacuoles were also found. Hemosiderin in great masses in the liver, veins of the liver, and about the kidney tubules was very prominent; so much was massed in the spleen that the cellular elements of the organ were obscured. The kidney of this specimen presented no signs of injury.

Series II (0.025%):

In this series there was little immediate effect on the well-being of the fish. After the second day they ate little and became lethargic.

At the 10 hour period one male appeared essentially normal and the only islet change detectable even with a high magnification systematic examination was an increased vascularity. In addition to a capillary dilation the other three fish showed cellular changes within the islet. In most of the cells the nuclei were so hyperchromatic that a reticulum was obscured. Granulation as seen with the azan stain did not seem to be altered. The ends of the beta cells had become rounded, thus giving the cells a more nearly rectangular shape. The kidney tubules were unimpaired histologically. The liver parenchyma appeared normal but an abnormally large amount of hemosiderin was in the organ.

After an elapse of 20 hours one male was found dead and was discarded because of advanced decomposition. In all killed specimens degenerate changes were now definite and more advanced than at the previous interval. Beta cell nuclei were pycnotic with no structure visible. Cytoplasmic degranulation was in progress; cells totally devoid of granules, as well as cells with a nearly normal amount of granules, were noted. In still other cells a clumping or coalescence of granules had taken place. Cellular shrinkage was so advanced that the individual cells in some sections were detached and separated from one another by large spaces. The mesonephros epithelium showed a slight reduction in height in three specimens, a male being the exception. Localized areas of cell shrinkage were present in the liver of a male and female. The amount of hemosiderin was greatly increased in the livers of all the specimens. This substance was also found around the kidney tubules.

One female fish died in the 30-44 hour interval; this was discarded because of decomposition. In all four animals sacrificed at the 44 hour period, the beta cell nuclei were hyperchromatic and caryorrhexis was evident in some sections. Degranulation was near the terminal stages and many cells totally devoid of granules were seen. With eosin the cytoplasm was clear and stained more homogeneously than at the earlier involution stages or in the normal cells. With azan stain many of the cells were colorless or pale blue. Cell shrinkage was so pronounced that the cells were separated from one another; in some areas they were arranged in irregular compact masses as in Fig. 9. The livers of the four fish showed small loci of cell shrinkage; the nuclei of these elements were normal. As previous, abnormally large amounts of hemosiderin were found in this organ. This substance was also present about the kidney tubules, the epithelium of which was reduced in height.

Beta cell caryorrhexis and cytolysis had taken place in the 44-70 hour interim. Nuclei, cell boundaries and granules were not found at this time. An acidophilic debris had replaced the cells. Alpha cells bordering these areas were swollen but presented no degenerate changes or mitotic figures. Lowering of kidney epithelium height was evident in all four animals. The livers were histologically the same as at 44 hours.

After 5 days neither intact beta cells nor an acidophilic debris were seen. Instead, spaces near the marginal border, the area formerly occupied by the beta cell columns, were observed. Kidney epithelium was now so reduced that it resembled the low cuboidal or the simple squamous type. This change is seen by comparing Fig. 12 with Fig. 13. In the lumen of a few tubules a pale-staining eosinophilic substance appeared. Slight vacuolations were noted in the livers of two fish, the other two presenting the cell shrinkage seen after 44 hours. The hemosiderin deposit was increased in all of the livers. This compound was also massed about the mesonephros tubules of one fish. In this specimen the splenic cells were masked by the great amount of hemosiderin deposited in the organ.

The spaces seen in the islet at the 5 day interval were still apparent on the seventh day. No multiplication or invasion of the area by other cell types had taken place. The histological structure of the kidney was about the same as at the 5 day interval, the only changes discernible being an increased amount of hemosiderin and the presence of an intralumen oxyphilic substance. Vacuolations and small localized lesions were apparent in the liver of one fish; small areas lacking intact cells were found throughout the livers of the other three.

At 9 days the histological picture of all organs was about the same as that seen at the seventh day. Abnormally large, dense black osmophilic droplets at the peripheral areas of the liver suggested that a fatty infiltration of this organ was taking place.

Series III (0.013%):

All fish swam about, took food and gave every evidence of being in good health until the third day. After that time little food was taken, the fish became quiescent and sought the corners of the tanks.

Of the four fish examined at the 10 hour interval, one female presented an essentially normal histological picture. As seen under low magnification the main islet change in the other three specimens was an increased blood supply. Capillary dilation was visible and erythrocytes were more abundant than normally found in the structure. A few hyperchromatic nuclei were observed with higher magnification. Beta cell granulation remained within the normal range. Both the kidney and the liver were unchanged in all specimens.

By 20 hours practically all of the beta cell nuclei of all the specimens were pycnotic. Capillary dilation was evident. High power examination revealed that the granulation of many cells was slight, but it could not be determined if it was still within the normal range or was an abnormality. The liver parenchyma of all fish was normal, a hemosiderin deposition greater than normal being noted in the gland. The kidneys presented no abnormalities.

At the 44 hour interval the beta cell nuclei were hyperchromatic. Caryolysis was not seen. With the azan stain a great diminution of granules was noted in many cells of three fish; the other fish, a male, presented a doubtful picture in this regard. The cytoplasm of the beta cells undergoing degranulation stained very pale with eosin. Cell shape alteration was in progress and many rounded cells were seen. The columnar arrangement of cells near the gall bladder was impaired and in some sections the elements were now in clusters. The kidney epithelium height was not reduced, but the cytoplasm stained lighter than normal. The livers of two fish (one male and one female) were normal except for a greater than normal deposition of hemosiderin. The parenchyma of the other livers had a looser arrangement than that regularly seen.

After 70 hours the beta cell nuclei were shrunken and deep-staining. Granulation was sparse and only a few granules could be seen in a small number of cells. In the degranulated cells the cytoplasm stained homogeneously and blue with the azan stain. Islet architecture was not within the normal range. Definite columns of cells were replaced by clusters of crumpled cells with pycnotic nuclei. The exocrine cells in all, except one fish (a male), were normal. In the latter the cytoplasm was degranulated and pale-staining. The kidneys of all fish presented the same details as at 44 hours. Three of the livers had some shrunken cells and a slight vacuolation; the other (from a female) was normal. All four showed an increased hemosiderin deposit in this organ and around the kidney tubules.

On the fifth day the marginal border of the islet which normally contains the beta cells, was without intact cells. An eosinophilic cellular debris and cytoplasmic masses without nuclei or cell boundaries were the only remains of cells to be found. The kidney epithelium was slightly reduced and vacuolated; the cytoplasm stained pale. An eosinophilic colloid-like substance was present in the lumen of the tubules. Although the liver parenchyma of all of the fish was shrunken, the nuclei of the hepatic cells were normal. Hemosiderin deposition in both the liver and the kidney was again greater than normal.

By the seventh day the beta cell debris was replaced by a fine connective tissue stroma. The alpha cells adjacent to the area were slightly swollen, but their granulation was not affected. Mitotic figures were not seen in the islet. The kidney epithelium of all specimens was reduced in height and the nuclei of these elements now were hyperchromotic. A granular and reticular material was noted in the lumen of the tubules. Cytoplasmic shrinkage and vacuolation was seen in varying degrees in all of the livers; in fact, areas consisting only of connective tissue were found. A great hemosiderosis was also present in the organ. These changes can be seen by comparing Fig. 10 and Fig. 11.

The main feature demonstrated on the ninth day was solid, dense black osmophilic droplets at the peripheral areas of the liver. These were present in all of the specimens.

The male and female fish that were maintained until death, died on the forty-first and thirty-ninth days respectively. During this time they were sluggish and took little food. At the time of death a loss of weight was apparent, their bodies had greatly thinned and attained a degree of transparency. Both fish were found dead and were not suitable for study because of decomposition.

Discussion.

That the teleostean fishes possess the homologue of the mammalian islands of Langerhans was postulated by Diamare in 1899. The studies of Massari (1898), Rennie (1903, 1905) and especially that of McCormick (1926) demonstrated that such structures are found in many different species of both fresh water and marine teleosts. As a rule the islets are fewer in number and are larger than those found in mammals. The number of islets varies with the species. For example, one is found in *Gadus callarias* Linnaeus, while about 50 are found in *Ameiurus lacustris* (Walbaum) (McCormick, 1926). When more than one islet exists it is usually noted that one of the number is bigger than any of the others and that it may in some cases be seen with the naked eye. Rennie (1903) termed the largest islet the "principal islet." In the present study it was found that the guppy has only one islet and that it can in large specimens be seen macroscopically.

TABLE I.

Histological Changes After Alloxan Administration.

	Series I. 0.05%	Series II. 0.025%	Series III. 0.013%
10 hours	Islet capillaries dilated. B nuclei hyperchromatic.	Islet capillaries dilated. B nuclei deep staining. Hemosiderin in liver.	Islet capillaries dilated.
20 hours	B nuclei condensed, some caryorrhexis. B cell degranulation and shrinkage in progress. Hemosiderin in liver and kidney.	B nuclei pycnotic. B cell degranulation and shrinkage in progress. Slight reduction of kidney epithelium height. Hemosiderin in liver and kidney.	B nuclei pycnotic. Hemosiderin in liver.
40 hours	B cell disintegration. Liver lesions. Hemosiderin in liver, kidney and spleen.		
44 hours		Some B nuclei fragmentation. Advanced B cell degranulation. Pronounced B cell shrinkage. Cell shrinkage in liver. Kidney epithelium height reduced. Hemosiderin in liver and kidney.	B nuclei pycnotic. B cell degranulation and shrinkage in progress. Hemosiderin in liver.
70 hours		B cell caryorrhexis and cytolysis. Kidney epithelium height reduced. Hepatic cell shrinkage. Hemosiderin in liver and kidney.	B nuclei pycnotic. B cell granulation sparse. B cell break-up. Liver vacuolated. Hemosiderin in liver and kidney.
5 days		No intact B cells. Marked reduction of kidney epithelium height, intralumen substance present. Cell shrinkage and vacuolation in liver. Hemosiderin in liver, kidney and spleen.	No intact B cells. Kidney epithelium height reduced with intralumen substance present. Hemosiderin in liver and kidney.
7 days		Marginal area of islet devoid of cells. Kidney epithelium height reduced. Lesions and vacuolations in liver Hemosiderin as at 5 days.	As at 5 days plus liver lesions.
9 days		Large osmophilic droplets in liver.	Large osmophilic droplets in liver.

Some early workers tried to relate the fish islet to the endocrine system and carbohydrate metabolism. Diamare in 1905 attempted the preparation of a glucose-repressing extract from the islet tissue of different species of fish. Years later MacLoed[2] (1922) and McCormick & Noble[3] (1925) succeeded in obtaining insulin from a variety of teleostei. Jackson (1922) demonstrated by the differential solubility method of Lane the presence in fish of two cell types having the same characteristics as the alpha and beta cells of the mammalian islands. Thus on both histological and physiological grounds it was shown rather conclusively that the islet of the teleost is represented in mammals by the islands of Langerhans. Bowie in 1924 continued and extended the cytological work by using a differential staining method; at that time he demonstrated three types of granular cells in the islet of *Neomaenis griseus*. He termed them alpha, beta and gamma cells. In the present report the existence of three types of granular cells is also recorded but the data are not in strict accord with that of Bowie's, the main difference being in the granular detail. Bowie claimed that the beta cell granules are larger than the alpha cell granules and that they may even approach the size of the zymogen granules of the exocrine pancreatic cells. The reverse situation was found to prevail in *Lebistes reticulatus*. Bowie's description of the gamma cell corresponds rather well with that given here for the "D" cell. If we allow for the fact that different techniques and a very different fish were used, it is possible that the same cell types are to be found in both *Neomaenis griseus* and *Lebistes reticulatus*.

Bensley (1911) noted that in addition to the alpha and the beta cells an agranular "C" cell is present in the guinea pig pancreas. For many years a controversy as to whether this "C" cell is a fundamental cell type or is peculiar to the guinea pig has been waged. In most of the more recent investigations the agranular cell has not been reported to exist in animals other than the guinea pig. In Bowie's report no mention was made of the "C" cell type. The present study, employing a differential staining method that is capable of demonstrating such a cell, failed to reveal any type of granular cell. The "D" cell type has been described in man (Bloom, 1931), in many other mammals and in the elasmobranchii (Thomas, 1937, 1940). It seems that the three granular cells, the alpha, the beta and the "D" cells, are the more fundamental islet components.

It is evident from the experimental results of this report that islet cell injury similar to that which has been reported to take place in mammals can be produced in the teleost *Lebistes reticulatus* by the immersion of the fish in an alloxan solution of suitable concentration. It is to be noted that the rate at which the beta cell cytological changes were produced and the time of death of the fish depended upon the alloxan dosage employed. There also exists an initial variation in the affectibility of individual fish to the same concentration of the drug. The deviations may be a reflection of a difference in the activity of the individual cells at the time they are first acted upon by the alloxan. In the series of experiments of this study several early deaths occurred when a concentration of 0.05% alloxan solution was used. It appears that this concentration is very close to, if not the actual upper sub-lethal dose for the guppy by the immersion method. The early deaths were not convulsive and from the actions of the fish in seeking the surface they may in part be attributed to an interference with respiration. It has been shown by Shipley & Rannefield (1945) and Houssay *et al* (1946) that repeated, small, sub-diabetogenic doses may produce islet necrosis similar to that caused by a large massive dose. The immersion method as used in this experiment could theoretically produce an additive effect. Therefore it is not possible to come to any conclusion as to whether or not the 0.013% alloxan series represents the lowest possible beta cell toxic concentration for *Lebistes reticulatus*.

It is of interest to note that the islet changes in the guppy, even with the highest drug concentration used, do not become apparent as soon as they do in the mammalian forms studied. In most mammals the injuries are rapidly produced. In many studies nuclear changes, cytoplasmic degranulation and cellular fragmentation have been reported to take place within a few hours (Duff & Starr, 1944; Dunn *et al*, 1944); in fact, Hughes *et al* (1944) claimed that the initial islet injury in the rat occurs within five minutes of the alloxan administration. In the present study similar changes, though not to such an advanced stage, were noted after a lapse of 20 hours at the highest concentration. Whether the lag is innate or is a reflection of slow assimilation because of the immersion method might possibly be determined by administering alloxan by injections. Seiden (1945) also noted a lag when the frog was used as an experimental animal; he administered alloxan by injections. It may well be that the longer interval between the drug administration and the produced necrotic effects are in some way a reflection of a lower metabolic rate of the cold blooded vertebrates.

Although all three concentrations eventually produced the same results, the lower concentration required a longer time to do so. This slower action provided more information and permitted a more detailed analysis of the involution stages; therefore the main interest centers about the fishes that survived at the lower concentrations. The earli-

[2] *Anguilla rostrata* (LeSueur), *Myoxocephalus octodecimspinosus* (Mitchill), *Myoxocephalus scorpius* (Linnaeus), *Lophius piscatorius* Linnaeus.

[3] *Polychius virens* (Linnaeus), *Gadus callarias* Linnaeus, *Melanogrammus aeglefinus* (Linnaeus), *Merluccius bilinearis* (Mitchill), *Hippoglossus hippoglossus* (Linnaeus), *Pseudopleuronectes americanus* (Walbaum), *Hemitripterus americanus* (Gmelin), *Cyclopterus lumpus* Linnaeus, *Macrozoarces americanus* (Bloch & Schneider).

est changes noted were an increased vascularity, a dilation of the islet capillaries and a hyperchromasia of the nuclei of the beta cells. These alterations were followed by a change in the degree of granulation and in the staining quality of the cytoplasm. Soon after degranulation had started cell shrinkage took place; the latter modification caused a disturbance of the columnar arrangement of the cells. Finally, complete caryolysis and cytolysis occurred. The time of death of the individual cell can not be determined with a great degree of certainty from this study, but it most probably took place at the time of cell shrinkage. The lack of both mitotic figures and cell proliferation in the islet indicates that the beta cell damage is irreversible.

It is well known, as indicated above, that insulin having physiological characteristics of mammalian insulin can be obtained from teleost islets. Having shown that the cell type which is generally conceded to be the producer of insulin is present in the guppy, and that these cells were destroyed, and knowing that a removal of the islet cells of the teleost results in a diabetic state (Simpson, 1926), we may assume that the fish in this experiment became diabetic.

A review of the literature shows that the consensus of opinion is that island injury by alloxan is restricted to the beta cells. Several investigators claim that some alpha cells may also degenerate. Saviano (1947b), using two different species of fishes, reported that the principal injury was suffered by the dark-staining cells (Mallory-Heidenhaim-Gomori stain). He termed this cell type the alpha cell. In the present experiment the elements staining dark are considered to be the beta cells and the lighter elements are termed alpha cells. This terminology is based on criteria of cytological details listed above and generally accepted; it is substantiated by the differential reaction of the cell types to alloxan. Although a slight swelling of the alpha cells adjacent to the area of the injured beta cells was observed in several preparations, no definite signs of degeneration of these cells were discernible. The marked difference in the reaction of the alpha and the beta cells to alloxan is significant, and may be considered an indication of a a fundamental difference in the composition and in the physiological role of the two cell types.

A histological deviation from the usual cellular detail of the exocrine pancreatic cells was noted in two of the fish examined. In both a degree of degranulation, with a resulting lighter and more homogeneous staining of the cytoplasm, had taken place. The nuclei of these cells were unchanged. The sections suggested a state of exhaustion similar to that produced by a period of high secretory activity, rather than cells undergoing a degenerative process.

It has been found that in some higher forms a large dose of alloxan may lead to severe acute lesions of the kidney. Vacuolation and desquammation of the tubules is common. Goldner & Gomori (1943) and Houssay (1946) likened the injury to that caused by mercury. There exists a relationship between the drug dose and the renal damage; often the nephrotoxic dose is greater than the minimum diabetogenic dose (Goldner & Gomori, 1943). By taking advantage of this relationship a diabetic state without renal damage may be produced in some forms. Fish in all three series of this experiment underwent kidney tubule modifications. A reduction in epithelial height with an increase in the lumen size (while the outside diameter of the tubule remained constant) was noted. An eosinophilic substance within the lumen was present in some specimens. These alterations are very similar to those described in studies of mammals. It is thought that the mammalian tubule damage is due to a direct sensitivity of the structures to the drug rather than being a consequence of islet cell alteration; for had the changes been caused by the produced permanent islet necrosis, it is not likely that the kidney would have reverted both functionally (Bennett & Behrens, 1946) and histologically (Ruben & Yardumian, 1946) to the normal state. In the present study the kidney remained histologically abnormal throughout the observed period, but it must be remembered that here, by the immersion method, the fish were constantly exposed to the drug and that even if the tubules are capable of repair they may be prevented from doing so by the uninterrupted exposure to the toxic substance.

Localized hepatic lesions in the form of cytoplasmic shrinkage, cellular disintegration and collapsed sinusoids were apparent in some preparations. An inflammatory reaction was not present. The livers of the specimens which were fixed with Flemming's fluid contained abnormally large black droplets suggestive of a fat infiltration. Because the normal liver contains much oil it is difficult to determine with osmic acid whether this represented an extreme fatty infiltration of the organ. The Bouin-fixed, hematoxylin-eosin stained liver tissue of fish killed at the later intervals presented large round vacuoles not seen in the normal liver; this is also suggestive of a fatty infiltration. The first mentioned lesions seem to be a direct alloxanic effect rather than a result of a possible diabetic state, for they developed in the time that the islet cells were still undergoing change. The fat infiltration was noted several days after the start of the experiment, therefore it can not be determined if the change was due primarily to alloxan or to a disturbed metabolism.

Another complication following the administration of alloxan is an increased hemosiderosis in some animals. Lowered erythrocyte counts, hemoglobinemia and hemoglobinuria were recorded to take place in rabbits (Kennedy & Lukens, 1944) and in rats (Gyorgy & Rose, 1948). Herbut *et al* (1946) noted a

hemochromatosis in rabbits after alloxan treatment. In the present study greater than normal iron deposits, indicative of hemosiderin, were deposited in the liver, kidney, large blood vessels and the spleen. Since no blood counts were made we must consider two hypotheses to explain the abnormally large amount of hemosiderin. First, the hemosiderin deposition is the resultant of a decrease in excretion of the compound due to an impairment of the excretory mechanism. Second, the accumulation results from an excessive production of erythrocyte breakdown products. The fact that the hemosiderin deposits were observed before the kidney and liver damage became apparent suggests that the latter hypothesis may explain the findings but does not completely eliminate the first one. It may well be that the deposition was at first due to an excessive hemolysis and that the condition was later aggravated by disturbed renal and hepatic function. The works of Kennedy & Lukens (1944) and of Gyorgy & Rose (1948) are excellent evidence for an increased hemolysis in the forms they studied. Kennedy & Lukens also reported that the effects are reversible. Gyorgy & Rose proved that in rats the hemoglobinuria which usually follows a diabetogenic dose of alloxan may be prevented by maintaining the animals on a diet rich in tocopherol. The increased hemolysis in the rat is therefore independent of the other effects of alloxan. Whether or not the same condition exists in the species of fish reported in this study can be determined only by further study.

The above reactions indicate that alloxan, or a produced derivative, is a multi-factor agent.

Summary.

1. The teleost *Lebistes reticulatus* has pancreatic endocrine epithelial cells condensed into one compact nodule.

2. The islet consists of three types of granular cells: the alpha, the beta and the "D" cells. The alpha cells are more abundant than the beta variety. The "D" cells are the least numerous. An agranular cell type is not present.

3. The beta cells of the islet undergo nuclear pycnosis, cytoplasmic degranulation and complete disintegration after the administration of alloxan. The alpha and the "D" cells are not subject to necrosis and remain undamaged.

4. After exposure to alloxan the kidney epithelium height is reduced; in consequence of this alteration the size of the lumen of the tubules is increased while the outside diameter of the structures remains normal.

5. Liver parenchymatous injury occurs after alloxan treatment. Necrosis and a degree of fat infiltration of the organ take place.

6. A large amount of hemosiderin is found intravsacularly and in the liver, kidney and spleen of the alloxan-treated fish.

7. No sex difference is noted for any of the above reactions.

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EXPLANATION OF THE PLATES.

All photomicrographs were taken of tissue fixed in Bouin's fluid; tissue represented by Figs. 4 and 5 were stained with azan, the other figures represent tissue stained with Harris' hematoxylin and eosin.

PLATE I.

Fig. 1. Parasagittal section of a normal fish showing the islet on the ventral surface of the stomach, anterior to the spleen. × 16.

Fig. 2. Frontal section of a normal fish. The islet is seen on the median line, close to the liver and anterior to the spleen. × 16.

PLATE II.

Fig. 3. Cross section of a normal fish. The islet is seen on the ventral surface of the stomach adjacent to the liver. × 25.

Fig. 4. Portion of the median border of the islet of a normal fish, consisting almost exclusively of beta cells. These cells are dark and so heavily granulated that the nucleus of some of the cells is not visible. × 1200.

Fig. 5. Portion of the median border of the islet of an alloxan-treated fish. The beta cells are considerably degranulated. × 1200.

PLATE III.

Fig. 6. Cross section of the islet of a normal fish. Exocrine pancreatic cells are about the islet. At the right of the islet, blood vessels and gall bladder epithelium are visible. × 340.

Fig. 7. Cross section of the islet of an alloxan-treated fish. At the right (the median border of the islet) shrinkage of the beta cells with an opening of spaces between cell cords is marked. A partial encirclement of the islet by pancreatic acinar cells is seen. × 340.

Figs. 8 & 9. Cross section of the islet of an alloxan-treated fish. At the right (the median border of the islet) beta cell degeneration is seen. The cell outlines are indistinct. The normal columnar arrangement of the cells is no longer visible. The cytoplasm is pale-staining and confluent. The greater part of the islet is unaffected; this area consists almost exclusively of alpha cells. Fig. 8, × 310; Fig. 9, × 525.

PLATE IV.

Fig. 10. Cross section of the liver of a normal fish. × 550.

Fig. 11. Cross section of the liver of an alloxan-treated fish, showing shrunken parenchyma cells and vacuolization. The blood vessel at the upper right contains hemosiderin. × 550.

Fig. 12. Cross section of the kidney of a normal fish. × 170.

Fig. 13. Cross section of the kidney of an alloxan-treated fish. The tubular epithelium is greatly reduced. In the lower right corner two masses of hemosiderin are seen. × 170.